Knobbe Martens Olson & Bear LLP
Intellectual Property Law

550 West C Street
Suite 1200
San Diego CA 92101
Tel 619-235-8550
Fax 619-235-0176
www.kmob.com

Daniel Hart, Ph.D.

February 8, 2006
VIA FACSIMILE AND INTERNATIONAL FEDERAL EXPRESS

European Patent Office
International Preliminary Examining Authority
D-80298 Munich
GERMANY

Attention: Authorized Officer Noë

Re: **Response to Written Opinion—PCT CHAPTER II**
Title: DEFINITIVE ENDODERM
Application No.: PCT/US2004/043696
Filed: December 23, 2004
Our Reference No.: CYTHERA.045VPC

Dear Officer Noë:

This letter is provided in response to the Written Opinion that was mailed November 8, 2005 in connection with the above-identified PCT application.

Pursuant to Article 34, please replace pages 52-58 with replacement pages 52-58. Claims 36, 63 and 64 have been amended. The numbering of the replacement claim set is identical to that of the original claim set. In addition to the replacement pages, Applicants provide herewith a marked up version of the claims showing the changes made.

Claims 1-17, 62, 74 and 75 are Novel

Reference D1

The Examining Officer asserts that claims 1-17, 62, 74 and 75 lack novelty in view of reference D1. In particular, it is alleged that reference D1 discloses cell cultures produced by the differentiation of human embryonic stem cells to endodermal cells by treatment with NGF or HGF.

Applicants respectfully submit that claims 1-17, 62, 74 and 75 are novel over reference D1 because, among other things, this reference does not disclose human **definitive endoderm** cell cultures. Claim 1 recites "[a] cell culture comprising human cells wherein at least about 10% of said human cells are **definitive endoderm** cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom." Reference D1 does not disclose such cell cultures.

Reference D1 purports to disclose a process for directing the differentiation of cells early in the developmental stage. In particular, reference D1 describes a process for producing human embryoid bodies, transferring the embryoid bodies to a solid substrate and allowing them to randomly differentiate so as to produce randomly differentiated embryonic cells (DE cells). The randomly differentiated embryonic cells are then further differentiated in the presence of a growth factor (see Figure 1A). Specifically, reference D1 discloses that cultures of randomly differentiated embryonic cells were incubated for ten days in the presence of one of the eight growth factors listed in Figure 3A. At the end of the incubation period, non-quantitative, reverse transcription PCR was used to compare the level of expression of the marker genes listed in Figure 3A between cells incubated in the presence of a growth factor and untreated control cells. Inspection of Figure 3A shows that there are six different markers listed under the heading ENDODERM.

As indicated in Figure 3A, the markers **albumin** and **α1AT** are markers associated with terminally differentiated liver cells. These markers may also be expressed in other cell types, but they are not markers of definitive endoderm. Similarly, the markers **amylase, PDX1** and **insulin** are markers of terminally differentiated pancreatic cells as well as certain other tissues, but they are not markers of definitive endoderm. Figure 3A erroneously indicates that the final-listed marker, **AFP** (α-fetoprotein), is a marker of both definitive endoderm and visceral endoderm (an extraembryonic endoderm). However, in the present specification, Applicants have demonstrated using quantitative PCR (Q-PCR) that the AFP gene is expressed in extraembryonic endoderm, such as visceral endoderm, but it is not substantially expressed in definitive endoderm (see the instant application at pages 46 and 47, paragraphs 205-208, including Table 1, Figure 13A and elsewhere throughout the specification). This property of definitive endoderm cell cultures is specifically recited in claim 74 of the instant application.

Further inspection of Figure 3A of reference D1 shows that, under the differentiation conditions used, cells treated with either NGF or HGF express significant quantities of AFP, indicating that the resulting cells were not definitive endoderm. Furthermore, inspection of Figure 3B indicates that, as compared to control cultures, AFP expression is increased in the presence of both NGF and HGF. Such results are consistent with the production of extraembryonic endoderm, which expresses AFP, but inconsistent with the production of definitive endoderm, which does not significantly express AFP.

In view of the foregoing, there is no evidence to suggest that the differentiation methods using NGF or HGF described in Reference D1 would produce any definitive endoderm cells. However, even if a small number of definitive endoderm cells were produced using such methods, it should be noted that claim 1 of the instant application requires that **at least about 10%** of the human cells in the cell culture are definitive endoderm cells. Accordingly, even if some small number of definitive endoderm cells were present in the cultures disclosed in D1, such cultures would not destroy the novelty of claim 1 or the claims depending therefrom.

Moreover, it is unlikely that any significant amount of definitive endoderm could be produced using any of the methods disclosed in reference D1 because the cells used as starting material are already substantially differentiated. As described above, rather than starting with

human embryonic stem cells, reference D1 discloses the use of randomly differentiated embryonic cells generated from embryoid bodies. According to Figure 4, incubation of such starting material with any growth factors other than NGF or HGF did not lead to the production of endodermal cells of any type. Thus, none of the cell populations produced in Reference D1 contained any significant quantity of definitive endoderm cells.

In view of the foregoing remarks, Applicants respectfully submit that claims 1-17, 62, 74 and 75 are novel over reference D1.

Reference D2

In addition to the objection of claims 1-17, 62, 74 and 75 based on reference D1, the Examining Officer asserts that reference D2 allegedly discloses all of the elements of these claims. Applicants would like to initially point out that reference D2 was first published subsequent to the earliest priority date of the instant PCT application. As the claims are fully supported by the priority application, Reference D2 does not qualify as prior art within the meaning of Rule 61.1(b).

Moreover, even if Reference D2 did qualify as prior art, it still does not disclose all of the limitations of claim 1. In particular, reference D2 does not disclose **human** cell cultures. Rather, all of the cell cultures described in reference D2 are derived from GFP-Bry mouse ES cell lines reported by Fehling et al. *Development* **130**: 4217-4227 (see reference D2 at page 1652, column 2, first full paragraph). Inspection of Fehling et al. shows that the ES cells used to establish the GFP-Bry ES cell line used in reference D2 were E14.1 ES cells derived from 129/Ola mice (see Fehling et al. at page 4218, column 2, first full paragraph). As such, reference D2 discloses only mouse cell cultures. It does not disclose human cell cultures as recited in claim 1.

In view of the foregoing remarks, Applicants respectfully submit that claims 1-17, 62, 74 and 75 meet the requirements of Article 33(2) PCT.

Claims 1-35, 62 and 73-75 are Inventive

The Examining Officer asserts that claims 20-35 and 73 lack an inventive step in view of reference D1. In formulating this objection, the Examining Officer relies on the alleged disclosure in D1 of the differentiation of human embryonic stem cells to endodermal cells by treatment with NGF or HGF. However, as discussed above, reference D1 does not disclose cell cultures of human definitive endoderm cells. Thus, the basis for this objection is not correct and the objection should be withdrawn.

Moreover, the Examining Officer relies on a separate incorrect premise in setting forth the grounds for the alleged lack of inventive step for Claim 20 and its dependent claims. The Examining Officer asserts that the problem of providing a cell population comprising at least about 90% human definitive endoderm cells could be solved by one or ordinary skill in the art by using known endodermal cell markers to purify the definitive endoderm cells from the cell cultures disclosed in reference D1. However, at the time of filing the instant application, it

would not have been possible for a skilled artisan to purify human definitive endoderm cells using human endodermal cell markers. At the time of filing the instant application, there were no human endodermal cell markers that were known to be specific for definitive endoderm. In other words, each of the known markers for human endodermal cells was expressed in a plurality of endodermal cell types, including extraembryonic endoderm.

The present invention includes Applicant's discovery of the first known markers specific for definitive endoderm cells that could be used in purification processes, such as those suggested by the Examining Officer. One marker of particular interest includes the chemokine receptor CXCR4. In particular, Applicants have discovered that CXCR4 is expressed on the surface of definitive endoderm cells present in definitive-endoderm-containing cell cultures differentiated from human embryonic stem cells, but it is not expressed in the other cell types present in such cultures, such as extraembryonic endodermal cells, embryonic stem cells and mesodermal cells. Using the CXCR4 marker, Applicants have been able to obtain definitive endoderm cell populations having greater than 98% purity (see Example 10 of instant application at pages 54 to 56). Accordingly, the enriched definitive endoderm cell populations described in independent claim 20, as well as claims depending therefrom, are inventive.

In view of the foregoing remarks, Applicants respectfully submit that claims 1-35, 62 and 73-75 meet the requirements of Article 33(3) PCT.

Each of the Claims Meets the Requirements of Article 6 PCT

The Term "About"

The Examining Officer asserts that the term "about" as used in claims 1-3, 14, 20-22, 33, 37-40. 53-58, 68 and 69 is vague and indefinite. However, the meaning of the term "about" can be determined from its usage in the specification as related to each of the claim terms that it modifies. For example, in claims 1-3, the usage of the term "about" relates to the percentage of human definitive endoderm cells in each human cell culture. In claims 53-58, the term "about" is used to describe the concentration of the growth factor provided in the cell culture. In each of these cases, the specification provides the context for interpretation of the term "about" (for example, see instant application at pages 24-25 and 31-32). As such, Applicants respectfully submit that use of the term "about" in claims 1-3, 14, 20-22, 33, 37-40. 53-58. 68 and 69 meets the requirements of Article 6 PCT.

Abbreviations

In addition to the foregoing objection, the Examining Officer asserts that the abbreviations of the marker genes recited in the claims should be explained in order to meet the requirement of Article 6 PCT. This Article requires that the claims clearly and concisely define the matter for which protection is sought. Whether a claim term is clear is judged by those of ordinary skill in the art.

Applicants agree that often the use of abbreviations in claims can sometimes lead to confusion that would be outside the scope of Article 6 PCT. However, in the case of the presently pending claims, the abbreviations that are used without an identifying longhand form are themselves are better understood by those having ordinary skill in the art than the longhand forms they represent. Indeed, Applicants have used the longhand forms of certain genetic markers where such longhand forms are understood by those of ordinary skill in the art. For example, "alpha-fetoprotein (AFP), Thrombomodulin (TM)" is recited in Claim 5. However, for the remainder of the markers recited in the claims, the abbreviations of gene names are the standard, unique gene symbols assigned by the Human Genome Organisation (HUGO) Gene Nomenclature Committee.

The HUGO symbols are the most widely used gene symbols in all fields related to molecular and cell biology. The corresponding longhand names of all of the markers can be identified by those of ordinary skill in the art at the Gene Nomenclature Committee's official website at http://www.gene.ucl.ac.uk/nomenclature/. However, these longhand names are far less familiar to those having ordinary skill in the art than the abbreviations recited in the claims. For example, the corresponding longhand gene name for SOX17, recited in Claims 5, 6 and 10, is "SRY (sex determining region Y)-box 17." This longhand name would not be immediately recognizable to those of ordinary skill in the art, while "SOX17" immediately conveys to those having ordinary skill in the art which gene is described.

One of the concerns of using abbreviations in claims rather than the full longhand versions is that the same abbreviations can represent more than one longhand term. Another such concern is that the abbreviations can change over time. However, neither of these concerns is applicable to the symbols used in the presently pending claims. As indicated on the homepage of the website indicated above under the heading "Advanced Gene Search," the HUGO Gene Nomenclature Committee ensures that "each symbol is unique and . . . that each gene is only given one approved gene symbol." Thus, each symbol is both unique and fixed over time.

Accordingly, at the time of filing the instant PCT application, one of ordinary skill in the art would be able to clearly recognize each marker gene recited in the claims simply from its unique HUGO gene symbol. The addition of the full name of each marker gene would actually distract from clarity because those of ordinary skill in the art are much more familiar with the HUGO gene symbol rather than the full gene name. As such, Applicants respectfully submit that use of the HUGO gene symbols **by themselves** in the claims most effectively meets **both** the **clarity** and **conciseness** requirements of Article 6 PCT.

Claim 63

The Examining Officer also asserts that claim 63 does not meet the requirement of Article 6 PCT. In particular, it is alleged that the technical feature necessary for solving the underlying problem is missing from the claim. Although claim 63 has been amended to include the step of providing a growth factor of the TGFβ superfamily, Applicants maintain that originally-filed claim 63 complies with Article 6 PCT.

Claims 36 and 64

Finally, the Examining Officer asserts that the phrase "in an amount sufficient to promote differentiation" as used in claims 36 and 64 is unclear under Article 6 PCT. Although claims 36 and 64 have been amended to remove the phrase "in an amount sufficient to promote differentiation," Applicants maintain that each of these claims are clear as originally written.

Conclusion

In view of the foregoing remarks, Applicants respectfully submit that each of the currently pending claims complies with Article 6 PCT.

Sincerely,

Daniel Hart
Reg. No. 40,637 (Agent of Record)

cc: International Docketing

Enclosures

2359055:020806

WHAT IS CLAIMED IS:

1. A cell culture comprising human cells wherein at least about 10% of said human cells are definitive endoderm cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom.

2. The cell culture of claim 1, wherein at least about 50% of said human cells are definitive endoderm cells.

3. The cell culture of claim 1, wherein at least about 80% of said human cells are definitive endoderm cells.

4. The cell culture of claim 1, wherein said definitive endoderm cells express a marker selected from the group consisting of SOX17 and CXCR4.

5. The cell culture of claim 4, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of OCT4, alpha-fetoprotein (AFP), Thrombomodulin (TM), SPARC and SOX7 in said definitive endoderm cells.

6. The cell culture of claim 4, wherein said definitive endoderm cells do not express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

7. The cell culture of claim 4, wherein said definitive endoderm cells express a marker selected from the group consisting of MIXL1, GATA4 and HNF3b.

8. The cell culture of claim 4, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

9. The cell culture of claim 1, wherein said definitive endoderm cells express SOX17 and CXCR4.

10. The cell culture of claim 9, wherein the expression of SOX17 and CXCR4 is greater than the expression of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

11. The cell culture of claim 9, wherein said definitive endoderm cells do not express OCT4, AFP, TM, SPARC and SOX7.

12. The cell culture of claim 9, wherein said definitive endoderm cells express MIXL1, GATA4 and HNF3b.

13. The cell culture of claim 9, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

14. The cell culture of claim 1, wherein at least about 2 definitive endoderm cells are present for about every 1 pluripotent cell in said cell culture.

15. The cell culture of claim 14, wherein said pluripotent cell comprises an embryonic stem cell.

16. The cell culture of claim 15, wherein said embryonic stem cell is derived from a tissue selected from the group consisting of the morula, the inner cell mass (ICM) of an embryo and the gonadal ridges of an embryo.

17. The cell culture of claim 1 further comprising a medium which comprises less than about 10% serum.

18. The cell culture of claim 1 further comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily.

19. The cell culture of claim 1, further comprising a growth factor selected from the group consisting of Nodal, Activin A, Activin B and combinations thereof.

20. A cell population comprising cells wherein at least about 90% of said cells are human definitive endoderm cells, said human definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom.

21. The cell population of claim 20, wherein at least about 95% of said cells are human definitive endoderm cells.

22. The cell population of claim 20, wherein at least about 98% of said cells are human definitive endoderm cells.

23. The cell population of claim 20, wherein said human definitive endoderm cells express a marker selected from the group consisting of SOX17 and CXCR4.

24. The cell population of claim 23, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said human definitive endoderm cells.

25. The cell population of claim 23, wherein said human definitive endoderm cells do not express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

26. The cell population of claim 23, wherein said human definitive endoderm cells express a marker selected from the group consisting of MIXL1, GATA4 and HNF3b.

27. The cell population of claim 23, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

28. The cell population of claim 20, wherein said human definitive endoderm cells express SOX17 and CXCR4.

29. The cell population of claim 28, wherein the expression of SOX17 and CXCR4 is greater than the expression of OCT4, AFP, TM, SPARC and SOX7 in said human definitive endoderm cells.

30. The cell population of claim 28, wherein said human definitive endoderm cells do not express OCT4, AFP, TM, SPARC and SOX7.

31. The cell population of claim 28, wherein said human definitive endoderm cells express MIXL1, GATA4 and HNF3b.

32. The cell population of claim 28, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

33. The cell population of claim 20, wherein at least about 2 human definitive endoderm cells are present for about every 1 pluripotent cell in said cell population.

34. The cell population of claim 33, wherein said pluripotent cell comprises an embryonic stem cell.

35. The cell population of claim 34, wherein said embryonic stem cell is derived from a tissue selected from the morula, the ICM of an embryo and the gonadal ridges of an embryo.

36. A method of producing definitive endoderm cells, said method comprising the steps of:

obtaining a cell population comprising pluripotent human cells;

providing said cell population with at least one growth factor of the TGFβ superfamily thereby promoting differentiation of said pluripotent cells to definitive endoderm cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom; and

allowing sufficient time for definitive endoderm cells to form, wherein said sufficient time for definitive endoderm cells to form has been determined by detecting the presence of definitive endoderm cells in said cell population.

37. The method of claim 36, wherein at least about 10% of said pluripotent cells differentiate into definitive endoderm cells.

38. The method of claim 36, wherein at least about 50% of said pluripotent cells differentiate into definitive endoderm cells.

39. The method of claim 36, wherein at least about 70% of said pluripotent cells differentiate into definitive endoderm cells.

40. The method of claim 36, wherein at least about 80% of said pluripotent cells differentiate into definitive endoderm cells.

41. The method of claim 36, wherein detecting the presence of definitive endoderm cells in said cell population comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

42. The method of claim 36, wherein detecting the presence of definitive endoderm cells in said cell population comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of AFP, TM, and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of AFP, TM, and SOX7 in said definitive endoderm cells.

43. The method of claim 42, wherein the expression of at least one of said markers is determined by Q-PCR.

44. The method of claim 42, wherein the expression of at least one of said markers is determined by immunocytochemistry.

45. The method of claim 36, wherein detecting the presence of definitive endoderm cells in said cell population comprises detecting the expression of at least one marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

46. The method of claim 36, wherein said at least one growth factor is of the Nodal/Activin subgroup of the TGFβ superfamily.

47. The method of claim 46, wherein said at least one growth factor is selected from the group consisting of Nodal Activin A, Activin B and combinations thereof.

48. The method of claim 47, wherein said at least one growth factor is Nodal.

49. The method of claim 47, wherein said at least one growth factor is Activin A.

50. The method of claim 47, wherein said at least one growth factor is Activin B.

51. The method of claim 36, wherein a plurality of growth factors of the TGFβ superfamily is provided.

52. The method of claim 51, wherein said plurality of growth factors comprises Nodal Activin A and Activin B.

53. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 10 ng/ml.

54. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 100 ng/ml.

55. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 500 ng/ml.

56. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 1000 ng/ml.

57. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 5000 ng/ml.

58. The method of claim 36, wherein said cell population is grown in a medium comprising less than about 10% serum.

59. The method of claim 36, wherein said pluripotent cells comprise stem cells.

60. The method of claim 59, wherein said pluripotent cells comprise embryonic stem cells.

61. The method of claim 60, wherein said embryonic stem cells are derived from a tissue selected from the group consisting of the morula, the ICM of an embryo and the gonadal ridges of an embryo.

62. A definitive endoderm cell produced by the method of claim 36.

63. A method of producing a cell population enriched in definitive endoderm cells, said method comprising the steps of:

providing a pluripotent human cell population with at least one growth factor of the TGFβ superfamily thereby producing definitive endoderm cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom;

providing to said cell population a reagent which binds to a marker expressed in said definitive endoderm cells but which is not substantially expressed in other cell types present in said cell population; and

separating said definitive endoderm cells bound to said reagent from said other cell types present in said cell population, thereby producing a cell population enriched in definitive endoderm cells.

64. The method of claim 63, wherein the differentiating step further comprises allowing sufficient time for definitive endoderm cells to form, wherein said sufficient time for definitive endoderm cells to form has been determined by detecting the presence of definitive endoderm cells in said cell population.

65. The method of claim 63, wherein detecting comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

66. The method of claim 63, wherein detecting comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of AFP, TM, and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the

expression of a marker selected from the group consisting of AFP, TM, and SOX7 in said definitive endoderm cells.

67. The method of claim 63, wherein detecting comprises detecting the expression of at least one marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

68. The method of claim 63, wherein at least about 95% of said cells are definitive endoderm cells.

69. The method of claim 63, wherein at least about 98% of said cells are definitive endoderm cells.

70. The method of claim 63, wherein said marker is CXCR4.

71. The method of claim 63, wherein said reagent is an antibody

72. The method of claim 71, wherein said antibody has affinity for CXCR4.

73. An enriched population of definitive endoderm cells produced by the method of claim 63.

74. The cell culture of any one of claims 4 or 9, wherein said definitive endoderm cells do not significantly express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

75. The cell population of any one of claims 23 or 28, wherein said definitive endoderm cells do not significantly express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

Abstract of the Disclosure

Disclosed herein are cell cultures comprising definitive endoderm cells and methods of producing the same. Also disclosed herein are cell populations comprising substantially purified definitive endoderm cells as well as methods for enriching, isolating and purifying definitive endoderm cells from other cell types.

MARKED UP VERSION OF CLAIMS SHOWING CHANGES

WHAT IS CLAIMED IS:

1. A cell culture comprising human cells wherein at least about 10% of said human cells are definitive endoderm cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom.

2. The cell culture of claim 1, wherein at least about 50% of said human cells are definitive endoderm cells.

3. The cell culture of claim 1, wherein at least about 80% of said human cells are definitive endoderm cells.

4. The cell culture of claim 1, wherein said definitive endoderm cells express a marker selected from the group consisting of SOX17 and CXCR4.

5. The cell culture of claim 4, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of OCT4, alpha-fetoprotein (AFP), Thrombomodulin (TM), SPARC and SOX7 in said definitive endoderm cells.

6. The cell culture of claim 4, wherein said definitive endoderm cells do not express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

7. The cell culture of claim 4, wherein said definitive endoderm cells express a marker selected from the group consisting of MIXL1, GATA4 and HNF3b.

8. The cell culture of claim 4, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

9. The cell culture of claim 1, wherein said definitive endoderm cells express SOX17 and CXCR4.

10. The cell culture of claim 9, wherein the expression of SOX17 and CXCR4 is greater than the expression of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

11. The cell culture of claim 9, wherein said definitive endoderm cells do not express OCT4, AFP, TM, SPARC and SOX7.

12. The cell culture of claim 9, wherein said definitive endoderm cells express MIXL1, GATA4 and HNF3b.

13. The cell culture of claim 9, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

14. The cell culture of claim 1, wherein at least about 2 definitive endoderm cells are present for about every 1 pluripotent cell in said cell culture.

15. The cell culture of claim 14, wherein said pluripotent cell comprises an embryonic stem cell.

16. The cell culture of claim 15, wherein said embryonic stem cell is derived from a tissue selected from the group consisting of the morula, the inner cell mass (ICM) of an embryo and the gonadal ridges of an embryo.

17. The cell culture of claim 1 further comprising a medium which comprises less than about 10% serum.

18. The cell culture of claim 1 further comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily.

19. The cell culture of claim 1, further comprising a growth factor selected from the group consisting of Nodal, Activin A, Activin B and combinations thereof.

20. A cell population comprising cells wherein at least about 90% of said cells are human definitive endoderm cells, said human definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom.

21. The cell population of claim 20, wherein at least about 95% of said cells are human definitive endoderm cells.

22. The cell population of claim 20, wherein at least about 98% of said cells are human definitive endoderm cells.

23. The cell population of claim 20, wherein said human definitive endoderm cells express a marker selected from the group consisting of SOX17 and CXCR4.

24. The cell population of claim 23, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said human definitive endoderm cells.

25. The cell population of claim 23, wherein said human definitive endoderm cells do not express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

26. The cell population of claim 23, wherein said human definitive endoderm cells express a marker selected from the group consisting of MIXL1, GATA4 and HNF3b.

27. The cell population of claim 23, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

28. The cell population of claim 20, wherein said human definitive endoderm cells express SOX17 and CXCR4.

29. The cell population of claim 28, wherein the expression of SOX17 and CXCR4 is greater than the expression of OCT4, AFP, TM, SPARC and SOX7 in said human definitive endoderm cells.

30. The cell population of claim 28, wherein said human definitive endoderm cells do not express OCT4, AFP, TM, SPARC and SOX7.

31. The cell population of claim 28, wherein said human definitive endoderm cells express MIXL1, GATA4 and HNF3b.

32. The cell population of claim 28, wherein said definitive endoderm cells express a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1.

33. The cell population of claim 20, wherein at least about 2 human definitive endoderm cells are present for about every 1 pluripotent cell in said cell population.

34. The cell population of claim 33, wherein said pluripotent cell comprises an embryonic stem cell.

35. The cell population of claim 34, wherein said embryonic stem cell is derived from a tissue selected from the morula, the ICM of an embryo and the gonadal ridges of an embryo.

36. A method of producing definitive endoderm cells, said method comprising the steps of:

obtaining a cell population comprising pluripotent human cells;

providing said cell population with at least one growth factor of the TGFβ superfamily ~~in an amount sufficient to promote~~ thereby promoting differentiation of said pluripotent cells to definitive endoderm cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom; and

allowing sufficient time for definitive endoderm cells to form, wherein said sufficient time for definitive endoderm cells to form has been determined by detecting the presence of definitive endoderm cells in said cell population.

37. The method of claim 36, wherein at least about 10% of said pluripotent cells differentiate into definitive endoderm cells.

38. The method of claim 36, wherein at least about 50% of said pluripotent cells differentiate into definitive endoderm cells.

39. The method of claim 36, wherein at least about 70% of said pluripotent cells differentiate into definitive endoderm cells.

40. The method of claim 36, wherein at least about 80% of said pluripotent cells differentiate into definitive endoderm cells.

41. The method of claim 36, wherein detecting the presence of definitive endoderm cells in said cell population comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a

marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

42. The method of claim 36, wherein detecting the presence of definitive endoderm cells in said cell population comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of AFP, TM, and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of AFP, TM, and SOX7 in said definitive endoderm cells.

43. The method of claim 42, wherein the expression of at least one of said markers is determined by Q-PCR.

44. The method of claim 42, wherein the expression of at least one of said markers is determined by immunocytochemistry.

45. The method of claim 36, wherein detecting the presence of definitive endoderm cells in said cell population comprises detecting the expression of at least one marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

46. The method of claim 36, wherein said at least one growth factor is of the Nodal/Activin subgroup of the TGFβ superfamily.

47. The method of claim 46, wherein said at least one growth factor is selected from the group consisting of Nodal Activin A, Activin B and combinations thereof.

48. The method of claim 47, wherein said at least one growth factor is Nodal.

49. The method of claim 47, wherein said at least one growth factor is Activin A.

50. The method of claim 47, wherein said at least one growth factor is Activin B.

51. The method of claim 36, wherein a plurality of growth factors of the TGFβ superfamily is provided.

52. The method of claim 51, wherein said plurality of growth factors comprises Nodal Activin A and Activin B.

53. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 10 ng/ml.

54. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 100 ng/ml.

55. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 500 ng/ml.

56. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 1000 ng/ml.

57. The method of claim 36, wherein said at least one growth factor is provided in a concentration of at least about 5000 ng/ml.

58. The method of claim 36, wherein said cell population is grown in a medium comprising less than about 10% serum.

59. The method of claim 36, wherein said pluripotent cells comprise stem cells.

60. The method of claim 59, wherein said pluripotent cells comprise embryonic stem cells.

61. The method of claim 60, wherein said embryonic stem cells are derived from a tissue selected from the group consisting of the morula, the ICM of an embryo and the gonadal ridges of an embryo.

62. A definitive endoderm cell produced by the method of claim 36.

63. A method of producing a cell population enriched in definitive endoderm cells, said method comprising the steps of:

~~differentiating cells in a population of pluripotent human cells so as to produce~~ providing a pluripotent human cell population with at least one growth factor of the TGFβ superfamily thereby producing definitive endoderm cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom;

providing to said cell population a reagent which binds to a marker expressed in said definitive endoderm cells but which is not substantially expressed in other cell types present in said cell population; and

separating said definitive endoderm cells bound to said reagent from said other cell types present in said cell population, thereby producing a cell population enriched in definitive endoderm cells.

64. The method of claim 63, wherein the differentiating step further comprises ~~obtaining a cell population comprising pluripotent human cells, providing said cell population with at least one growth factor of the TGFβ superfamily in an amount sufficient to promote differentiation of said pluripotent cells to definitive endoderm cells, said definitive endoderm cells being multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom, and~~ allowing sufficient time for definitive endoderm cells to form, wherein said sufficient time for definitive endoderm cells to form has been determined by detecting the presence of definitive endoderm cells in said cell population.

65. The method of claim 63, wherein detecting comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population,

wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

66. The method of claim 63, wherein detecting comprises detecting the expression of at least one marker selected from the group consisting of SOX17 and CXCR4 and at least one marker from the group consisting of AFP, TM, and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of SOX17 and CXCR4 is greater than the expression of a marker selected from the group consisting of AFP, TM, and SOX7 in said definitive endoderm cells.

67. The method of claim 63, wherein detecting comprises detecting the expression of at least one marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 and at least one marker from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in cells of said cell population, wherein the expression of a marker selected from the group consisting of FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 is greater than the expression of a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7 in said definitive endoderm cells.

68. The method of claim 63, wherein at least about 95% of said cells are definitive endoderm cells.

69. The method of claim 63, wherein at least about 98% of said cells are definitive endoderm cells.

70. The method of claim 63, wherein said marker is CXCR4.

71. The method of claim 63, wherein said reagent is an antibody

72. The method of claim 71, wherein said antibody has affinity for CXCR4.

73. An enriched population of definitive endoderm cells produced by the method of claim 63.

74. The cell culture of any one of claims 4 or 9, wherein said definitive endoderm cells do not significantly express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

75. The cell population of any one of claims 23 or 28, wherein said definitive endoderm cells do not significantly express a marker selected from the group consisting of OCT4, AFP, TM, SPARC and SOX7.

Abstract of the Disclosure

Disclosed herein are cell cultures comprising definitive endoderm cells and methods of producing the same. Also disclosed herein are cell populations comprising substantially purified definitive endoderm cells as well as methods for enriching, isolating and purifying definitive endoderm cells from other cell types.